12.0 MINIMUM PROCEDURAL STANDARDS FOR *IN VITRO* ER BINDING ASSAYS AND RECOMMENDATION OF SUBSTANCES FOR USE IN VALIDATION STUDIES

12.1 Introduction

Although published studies on the ability of substances to bind *in vitro* to the ER are relatively numerous, there are no published standard test guidelines for conducting such studies, and no formal validation studies have been performed to assess the reliability or performance of ER binding assays. To support the further standardization and validation of *in vitro* ER binding assays, minimum procedural standards for such assays and a recommended list of test substances for use in validation studies are provided. The minimal procedural standards and recommended test substances are based on a comparative evaluation of the 14 *in vitro* ER binding assays summarized and evaluated in this BRD (**Sections 6** and **7**). The RUC assay, which has been the most widely used method for identifying substances with ER binding activity, is proposed as the standard against which new tests should be evaluated.

12.2 Minimum Procedural Standards

12.2.1 Animal Studies

All studies utilizing animals should be approved by the Institutional Animal Care and Use Committee (IACUC) or its equivalent.

12.2.2 Dissociation Constant (K_d) of the Reference Estrogen

Irrespective of the source of the ER used, the dissociation constant, K_d, of the reference estrogen (e.g., 17β-estradiol) must be determined each time the assay is performed. The purpose of determining K_d is to demonstrate that the assay system is valid (e.g., a finite number of high affinity receptors are saturated with ligand) and to optimize the system with respect to receptor and ligand concentration. The K_d is determined in a saturation binding experiment that involves adding increasing concentrations of the radiolabeled reference estrogen to the ER preparation and measuring binding to the ER (Motulsky, 1995). To calculate specific binding of the radiolabeled reference estrogen to the ER, nonspecific binding is measured at each radioligand concentration by the addition of a nonlabeled estrogen at a concentration that occupies all available receptors. The nonspecific binding is then subtracted from the total binding (in the

absence of nonlabeled compound) of the radiolabeled reference estrogen (Motulsky, 1995). The K_d of the reference estrogen, which reflects its affinity for the specific ER preparation, can then be calculated, and is used to determine the appropriate concentration of reference estrogen to be used in competitive binding assays. To determine the K_d, the ER must be exposed to the reference estrogen at concentrations spanning five to six orders of magnitude.

12.2.3 Preparation of Test Substances

Test substances must be dissolved in water or in a solvent that is miscible with water. For substances not sufficiently water soluble, absolute ethanol, or DMSO are proposed as solvents. Preference is given to ethanol since this solvent has been used in most of the studies conducted to date. Other solvents may be used as long as it can be demonstrated that they do not interact with the test system. A solvent control set of assay tubes must be included in each assay. It might be necessary to characterize the solubility of the test substance in several solvents to identify the optimal solvent to use in the ER binding assay.

12.2.4 Concentration Range of Test Substances

To minimize effort and costs in screening/testing, and in recognition that adding excessive amounts of a test substance can perturb the test system through physicochemical mechanisms, most testing schemes include a limit dose (i.e., the highest dose that should be tested in the absence of solubility constraints). An agreed upon limit dose for *in vitro* ER binding screening assays has not been established. Historically, the highest dose tested in such assays has ranged generally from 1 to 100 μM, with some tests conducted at doses as high as 1 mM. The IC_{50} values (and thus the RBA values) reported for substances tested in various *in vitro* ER binding assays cover six orders of magnitude below the IC_{50} for 17β-estradiol, the reference estrogen. In the RUC assay, the median IC_{50} for 17β-estradiol is 3.8 nM. Thus, if testing for ER binding substances requires the ability to detect substances with an IC_{50} that is at least six orders of magnitude lower than that of 17β-estradiol, then the limit dose (unless precluded by chemical properties such as solubility) should be above 4 mM (e.g., 10 mM) to allow for the detection of an IC_{50} in the concentration range of interest. However, if five orders of magnitude are sufficient for RBA values, then the limit dose would have to be above 400 μM (e.g., 1 mM). Decreasing

the limit dose to 100 μM would limit the sensitivity of the assay to RBA values that cover approximately four orders of magnitude.

For the purpose of screening, it is proposed that the limit dose be 1 mM and that a concentration range from 1 mM to 1 nM, in 10-fold increments, be used. However, if it is suspected that the test substance may bind more strongly to the ER than 17β-estradiol, the dose range should extend from 10 pM to 10 μM in 10-fold increments.

For relatively insoluble substances, the highest dose should be at the limit of solubility and the concentrations tested should be in 10-fold increments. Testing at concentrations that precipitate in the test medium should be avoided to minimize false positive results associated with the non-specific interaction of the precipitate with the ER (Gray et al., 1997).

12.2.5 Solvent and Positive Controls

Concurrent negative, solvent, and positive controls must be included in each experiment. The negative control contains all the reagents of the test system, except the assay solvent, which is replaced with a known nonreactive material, such as water. This sample is processed with treated samples and other control samples to ensure the solvent does not interact with the test system. The solvent control consists of all the reagents of the test system, including the solvent, and should be tested at the highest concentration that is added with the test substance. A positive control substance is included to demonstrate the sensitivity of each experiment and to allow for an assessment of variability in the conduct of the assay across time. The volume of materials in the ER assay control tubes should equal that of ER assay tubes containing test substance and reference estrogen. Since the RBA for the reference estrogen, 17β-estradiol, is set at 100, it is recommended that a substance (e.g., tamoxifen, coumestrol) that induces an RBA value between two and three orders of magnitude lower be used as the positive control. The median RBA values of tamoxifen and coumestrol in the RUC assay are reported to be 3.1 and 1.9, respectively (**Appendix D**). If metabolic activation is included in the experimental protocol, then a positive control requiring metabolic activation will need to be included in each experiment to demonstrate the adequacy of the exposure conditions. An appropriate positive control for such studies has not yet been identified.

12.2.6 Within-Test Replicates

The IC_{50} value of the reference compound (i.e., 17β-estradiol), the positive control, and each test substance should be based on triplicate measurements at each dose level.

12.2.7 Dose Spacing

Generally, to obtain a binding curve, the concentrations of the reference estrogen and the test substances should be spaced by one order of magnitude (i.e., 1 nM, 10 nM, etc.) over the concentration range of interest (1 nM to 1 mM). This results in testing seven concentrations of the test substance in each test. If the range of doses is reduced, then equivalent spacing (e.g., half-log doses) of the seven doses over the smaller dose range should be used.

12.2.8 Data Analysis

Following the measurement of saturation binding of radiolabeled 17β-estradiol to the ER, and after correcting for nonspecific binding, the binding of 17β-estradiol is plotted against the log of the concentration of radiolabeled 17β-estradiol. The curve is analyzed with nonlinear regression techniques to determine B_{max} and K_d. Although a Scatchard analysis (Scatchard, 1949) is frequently used to obtain the K_d, this method has many disadvantages and is not recommended as the primary method (see **Section 2**). Competitive binding experiments use a constant concentration of radiolabeled 17β-estradiol to measure its displacement from the ER by varying concentrations of reference estrogen or test substance. These data are analyzed by nonlinear regression analysis to determine the IC_{50} of the test substance or the reference estrogen. The RBA value for the test substance is calculated by dividing the IC_{50} for 17β-estradiol (or other reference estrogen) by the IC_{50} of the test substance and multiplying the result by 100. The K_i is calculated using the Cheng-Prusoff equation (Cheng and Prusoff, 1973) as a means of assessing the reproducibility of the data from experiment to experiment.

$$K_i = \frac{IC_{50}}{1 + \frac{[\text{Radiolabeled } 17\beta\text{ - estradiol}]}{K_d}}$$

12.2.9 Assay Acceptance Criteria

An assay will be considered acceptable for evaluation if the following conditions are met:

- The unlabeled 17β-estradiol standard curve demonstrates that increasing concentrations of unlabeled 17β-estradiol can displace ^{3}H-17β-estradiol, and that the IC_{50} value for 17β-estradiol is approximately equal to the molar concentration of ^{3}H-17β-estradiol plus the K_d (determined by nonlinear regression and viewed by a Scatchard plot);
- The K_d and IC_{50} values for the unlabeled 17β-estradiol standard curve are within the confidence limits for historical data;
- The ratio of total binding in the absence of competitor to the amount of ^{3}H-17β-estradiol added per assay tube is not greater than 10%;
- The K_i, IC_{50}, and RBA values for the concurrent positive control are within the confidence limits for historical data; and
- The solvent control, at the concentration used, did not alter the sensitivity or reliability of the assay.

12.2.10 Evaluation and Interpretation of Results

A substance is classified as positive for binding to the ER if an IC_{50} value can be obtained and an RBA can be calculated. If an IC_{50} cannot be obtained after testing to the limit dose or the highest dose possible, the test substance is usually classified as being "negative" for *in vitro* ER binding. However, due to solubility constraints (for example), some test substances might induce a significant reduction in binding without achieving at least a 50% reduction in the binding of the reference estrogen to the ER. Until additional information becomes available about the significance of this category of dose response curves, such responses should be noted and the substances classified appropriately (e.g., "equivocal") for the test.

12.2.11 Test Report

At a minimum, the test report must include the following information:

Test substance:

- Name, chemical structure, and CASRN, if known;
- Physical nature (solid or liquid), and purity, if known; and
- Physicochemical properties relevant to the study (e.g., solubility, stability, volatility).

Solvent:

- Justification for choice of solvent if other than water or ethanol; and
- Information to demonstrate that the solvent, if other than an established solvent, does not bind to, or otherwise affect, the ER.

Estrogen receptor:

- Type and source of ER (if from a commercial source, the supplier must be identified);
- Isolation procedure or method for making construct if isolated protein used;
- Protein concentration of ER preparation; and
- Method for storage of ER, if applicable.

Test conditions:

- K_d of the reference estrogen;
- Rationale for the concentration of the reference estrogen;
- Composition of buffer(s) used;
- Concentration range of test substance, with justification;
- Volume of vehicle used to dissolve the test substance and the volume of test substance added;
- Incubation time and temperature;
- Type and composition of metabolic activation system, if added;
- Concentration range of positive and solvent/vehicle controls;
- Method used to separate free reference estrogen, if applicable;
- Method for analyzing bound reference substance;
- Methods used to determine K_i and IC_{50} values; and
- Statistical methods used, if any.

Results:

- Extent of precipitation of test substance;
- The solvent control response compared to the negative control;
- IC data for each replicate at each dose level for all substances, including confidence levels or other measure of intra-dose repeatability;

- Calculated K_i and IC_{50} values and confidence limits for 17β-estradiol, the positive control, and the test substance; and
- Calculated RBA values for the positive control and the test substance.

Discussion of the results:

- Historical K_i and IC_{50} values for the reference estrogen, including ranges, means, and standard deviations;
- Reproducibility of the K_i and IC_{50} values of the reference estrogen, compared to historical data;
- Historical solvent and positive control data with ranges, means, and standard deviations;
- Reproducibility of the K_i and IC_{50}/RBA values for the positive control substance, compared to historical data; and
- The nature of the binding dose response relationship for the test substance.

Conclusion:

- Classification of test substance with regard to *in vitro* ER binding activity.

12.2.12 Replicate Studies

Generally, replicate studies are not mandated for screening assays. However, in situations where questionable data are obtained (i.e., the IC_{50} value is not well defined, "equivocal" results are obtained), additional testing using a more narrow range of test substance concentrations to clarify the results of the primary test would be prudent.

12.3 Standardization of ER Binding Assays for Validation

Appendix B provides *in vitro* ER binding assay protocols (or standard operating procedures) provided by five investigators and one protocol for use with a commercially available ER binding test kit (Pan Vera Corporation, Madison, WI, USA). The assay protocols (as titled by the investigator) included in **Appendix B** are:

- The Estrogen Receptor Competitive Binding Assay Using Rat Uterine Cytosol, as provided by Dr. Susan Laws, U.S. EPA, NHEERL, Research Triangle Park, NC, and Mr. Gary Timm, U.S. EPA, Washington, DC.

- The Competitive ER Binding MCF-7 Whole Cell Assay, as provided by Dr. Guy Leclercq, Clinique et Laboratoire de Cancerologie Mammaire, Centre des Tumeurs de l'Universite Libre de Bruxelles, Brussels, Belgium.
- The Fluorescence Polarization Assay of the Competitive Binding of Ligands to Estrogen-Receptor Complexes, as provided by Dr William Allworth, Department of Chemistry, University of New Orleans, New Orleans, LA.
- The Competitive Ligand Binding Assay, as provided by Dr. Timothy Zacharewski, Dept. of Biochemistry, Michigan State University, Lansing, MI.
- The Rat Estrogen Receptor Equilibrium Exchange Assay, as provided by Dr. Weida Tong, Division of Genetic and Reproductive Toxicology, National Center for Toxicological Research, Jefferson, AR.

Inspection of these protocols provides a perspective on how various assays are conducted by different investigators.

12.3.1 Example Recommended General Protocol for Measuring ER Binding Using the RUC Assay

An example *in vitro* ER binding assay test method protocol is provided in the **Annex** to **Section 12** (designated Annex protocol). This recommended general (as opposed to laboratory-specific) protocol for the RUC assay is based on the RUC assay protocol supplied by the U.S. EPA (**Appendix B-5**) and on information obtained from expert U.S. EPA scientists (Drs. S. Laws, R. Cooper, E. Gray) and professional (Drs. J. Pounds, J. Morris) and technical staff at Battelle Pacific Northwest Laboratories. This general protocol takes into account the minimum procedural standards described in **Section 12.2**. Specific differences between the original U.S. EPA protocol and the version provided in the Annex are described in the following sections. The protocol is included solely to provide guidance to investigators interested in developing comparable laboratory-specific protocols; it has not been used to generate experimental data.

12.3.2 Preparation of Rat Uterine Cytosol (Annex Section 3, Appendix B-4 Section I)

- Animal Use: Consistent with U.S. Government policy, a statement has been added to the Annex protocol that all studies utilizing animals should be approved by the Institutional Animal Care and Use Committee (IACUC) or its equivalent.

12.3.3 Standardization of *In Vitro* ER Binding Assays (Annex Section 4, Appendix B-4 Section II)

- Data Analysis: Since the IC_{50} value is a property of the experiment and the K_i a property of the receptor and the test substance, the Annex protocol recommends that the K_i value be calculated and provided, in addition to the RBA value. Calculation and analysis of the K_i value is not considered in the U.S. EPA RUC protocol.
- Standardization Acceptance Criteria: The Annex protocol includes an assessment against published and historical data of the K_i, as well as the more typical IC_{50} values, for unlabeled 17β-estradiol. The K_i value is not considered in the U.S. EPA RUC protocol.
- Standardization Substances: The U.S. EPA RUC protocol proposes that DES, estrone, and ethinyl estradiol be used as positive and R1881 as negative ER binding substances during efforts to standardize the performance of the RUC assay in the laboratory. After consideration of the data provided in **Appendix D** (see **Table 12-1**), the Annex protocol proposes that tamoxifen and coumestrol be used as positive and β-sitosterol as negative ER binding substances for this purpose. Selection of these two positive ER binding substances is based on a desire to use substances that induce an RBA value between two and three orders of magnitude lower than the reference estrogen to assure the sensitivity of the assay. β-Sitosterol was selected as the negative ER binding substance because of the extent of available data (**Table 12-1**). The Annex protocol includes an assessment, where feasible, against published and historical data of the K_i, as well as IC_{50} and RBA values, for these substances.

12.3.4 *In Vitro* ER Competitive Binding Assay Methodology (Annex Section 5, Appendix B-4 Section III)

- Replicate Assay Tubes: The Annex RUC protocol specifies the use of triplicate (rather than duplicate) assay tubes per concentration tested. The additional assay tube will increase the accuracy of each measured response, and thus the accuracy of the calculated IC_{50} and RBA values.
- Solvent and Positive Controls: The Annex RUC protocol states that when testing substances for their ability to bind to the ER, concurrent negative, solvent and positive controls should be included in each experiment. The U.S EPA RUC protocol includes a solvent control and a

substance without ER binding activity as a negative control substance (tested at a single maximal concentration), but does not include negative or positive controls. A positive control substance is included in the Annex RUC protocol to demonstrate the sensitivity of each experiment and to allow for an assessment of variability in the conduct of the assay across time. While the reference estrogen provides some aspects of a positive control (i.e., it demonstrates the functionality of the assay), it does not allow for an evaluation of the variability in RBA values across experiments. The Annex RUC protocol does not recommend the routine use of a negative control substance.

- Stock Solutions: The Annex RUC protocol specifies that test substances be dissolved in water or in a solvent that is miscible with water. For substances not sufficiently water soluble, absolute ethanol or DMSO are proposed as solvents. Other solvents may be used as long as it can be demonstrated that they do not interact with the test system. The U.S. EPA RUC protocol specifies the use of absolute ethanol only. Choice of solvent should depend on which solvent allows the maximum testable concentration of the test substance.
- Serial Dilutions: In the Annex RUC protocol, it is proposed for the purpose of screening for ED substances that the limit dose be 1 mM. This limit dose (unless precluded by solubility constraints) allows for the detection of an IC_{50} value up to five orders of magnitude below that for 17β-estradiol, the reference estrogen. The U.S EPA RUC protocol specifies an upper limit dose of 0.3 mM.
- Evaluation and Interpretation of Results: In the Annex protocol, criteria for specifying a test substance as positive, negative, or equivocal for binding to the ER are provided. The U.S. EPA RUC protocol provides more limited guidance and does not consider the possibility of "equivocal" responses.
- Test Report: The Annex protocol specifies the information to be included in the Test Report; the U.S. EPA RUC protocol does not. Such guidance ensures that the test reports contain all pertinent information.
- Replicate Studies: The Annex protocol specifies situations for conducting replicate studies (i.e., in situations where questionable data are obtained) to clarify the results of the primary test . The U.S. EPA RUC protocol does not address the issue of replicate studies.

12.4 Recommended List of Substances to be Used for Validation of *In Vitro* ER Binding Assays

Table 12-1 provides a recommended list of substances to be used in the assessment of the reliability and comparative performance of existing or new *in vitro* ER binding assays. A number of factors were considered in developing this list, including the number of times the substance had been tested in the RUC assay, the median RBA value of the substance in the RUC assay, and the extent of concordance of the RUC median RBA value with values obtained for the same substance in other *in vitro* ER binding assays. Because the number of substances tested by multiple laboratories in the RUC assay was insufficient to generate the desired number of substances for consideration, selection of additional substances was based on the availability and concordance of multiple test data among the 13 other *in vitro* ER binding assays considered in this BRD, and the resulting median RBA value across assays. The selected substances were sorted according to their median RBA values. Because the spread of values extended over seven orders of magnitude, ranging from 400 to 0.0001, the substances were sorted into six categories in log decrements: $\geq$10, <10-1; <1-0.1; <0.01-0.1, <0.01-0.001; <0.001. Weakly-binding substances (RBA values <0.001) were difficult to identify because they were not always consistently positive in tests within an assay or between different assays. Also included were substances classified as "negative" for ER binding based on the lack of a positive response in multiple assays when tested at dose levels of at least 1 mM in at least one assay.

Table 12-1 Recommended Substances for Validation of *In Vitro* ER Binding Assays

Classification RBA Range	Substance	CASRN	Median RBA Value	Chemical Class	No. Assays in which Tested[a]	No. Assays with a Positive[a]
	DES	56-53-1	200*	Stilbene	14	14
	4-Hydroxytamoxifen	68047-06-3	175*	Triphenylethylene	13	13
	Estrone	53-16-7	48*	Steroid, phenolic	13	13
	Zearalenone	17924-92-4	44*	Acid lactone	10	10
	Estriol	50-27-1	14*	Steroid, phenolic	12	12
<10 to 1	2',4',6',-Trichloro-4-biphenylol	14962-28-8	3.6**	PCB	4	4
	Tamoxifen	10540-29-1	3.1*	Stilbene	14	14
	Bisphenol C2	14868-03-2	2.6*	Diphenylalkane	3	3
	Coumestrol	479-13-0	1.9*	Benzopyrone	11	11
	Mestranol	72-33-3	1.3*	Steroid, nonphenolic	2	2
<1 to 0.1	Nafoxidine	1845-11-0	0.72**	Triphenylethylene	6	5
	Genistein	446-72-0	0.56*	Flavone	11	11
	Norethynodrel	68-23-5	0.22*	Steroid, nonphenolic	3	3
	4-*tert*-Octylphenol	140-66-9	0.20*	Phenol	9	9
	Phloretin	60-82-2	0.069*	Flavone	3	3
<0.1 to 0.01	Bisphenol A	80-05-7	0.056*	Diphenylalkane	12	12
	Kepone	143-50-0	0.027*	Organochlorine	10	9
	Kaempferol	520-18-3	0.025*	Flavone	3	3
	5α-Dihydrotestosterone	521-18-6	0.014*	Steroid, nonphenolic	9	9

Classification RBA Range	Substance	CASRN	Median RBA Value	Chemical Class	No. Assays in which Tested[a]	No. Assays with a Positive[a]
	o,p'-DDT	789-02-6	0.013*	Organochlorine	12	10
<0.01 to 0.001	Naringenin	480-41-1	0.008*	Flavone	8	6
	4-Androstenedione	63-05-8	0.007**	Steroid, nonphenolic	3	1
	4-Chloro-4'-biphenylol	28034-99-3	0.007*	PCB	2	2
	4-Octylphenol	1806-26-4	0.005*	Phenol	5	4
	Methoxychlor	72-43-5	0.001*	Organochlorine	9	5
<0.001 to 0.0001	4-*tert*-Butylphenol	98-54-4	0.0009*	Phenol	1	1
	Morin	480-16-0	0.0005*	Flavone	1	1
	p,p'-DDT	50-29-3	0.0003*	Organochlorine	6	2
	Progesterone	57-83-0	0.0003*	Steroid, nonphenolic	2	1
	Atrazine	1912-24-9	0.0003*	Aromatic amine	6	1
Negative	Simazine	122-34-9	HTD-2000 µM	Triazine	6	0
	β-Sitosterol	83-46-5	HTD-1000 µM	Steroid, nonphenolic	8	0
	Diethylhexyl phthalate	117-81-7	HTD-5000 µM	Phthalate	1	0

Abbreviations: RUC = Rat uterine cytosol, DES = diethylstilbestrol; PCB = polychlorinated biphenyl; DDT = dichlorodiphenyltrichloroethane; HTD= Highest tested dose

[a]Negative test results at maximum tested concentrations <100 µM were excluded from consideration.

*Median RBA value for positive RUC tests

**Not tested in RUC, median RBA value across all other assays (positive tests only)

Five substances were selected for each RBA category and three for the negative category group. To ensure that each RBA category contained a representative sampling of chemical classes, selection was based on the chemical class to which the substance belongs and whether it was representative of a chemical class used in commerce or found in the environment, and whether it is commercially available. The latter criterion was based on whether the substance could be located in a chemical supply catalogue.

The chemical classes of the substances and the number of substances in each class in **Table 12-1** include nonphenolic steroids (6), organochlorines (4), polychlorinated biphenyls (PCBs), including hydroxylated derivatives (2), flavones (5), phenolic steroids (2), phenols (3), diphenylalkanes (2), stilbenes (2), triphenylethylenes (2), an aromatic amine (1), an acid lactone (1), a benzopyrone (1), a phthalate (1), and a triazine (1).

In March 2001, the U.S. EPA provided a list of 25 substances proposed for testing by Battelle Pacific Northwest (Richland, Washington) in an *in vitro* ER binding RUC assay procedure. In January 2002, EPA provided a modified list of 22 substances. Data generated by the U.S. EPA-sponsored study will be used to validate two QSAR models presently being developed by scientists at the FDA NCTR and by Dr. Mekenyan in Bulgaria. The 22 substances were chosen based on the availability of historical data demonstrating the *in vitro* ER binding affinity, ease of purchase at a purity of >98%, and the lack of extensive health and safety requirements for use (S. Laws, personal communication). Representation of all chemical classes was not a high priority.

The range of binding affinity for the chemicals included those expected to be high affinity binders (nM) to low affinity binders (μM and mM) to non-binders. The substances on the U.S. EPA list (**Table 12-2**) were compared to those recommended here. The U.S. EPA list lacks substances in certain chemical classes, such as PCBs and organochlorines, which have been demonstrated to bind to the ER (**Appendix D**). Since these two chemical classes are ubiquitous in the environment, representative substances were included in the list of substances recommended for validation in this BRD. However, due to possible concern

Table 12-2 List of Substances Being Tested in the *In Vitro* RUC Assay by Battelle

Classification RBA Range	Substances	RUC Median RBA Value	No. Times Tested in RUC Assay	Included in Recommended List in BRD
≥ 10	Meso Hexestrol	300	2	No
	17α-Ethinyl estradiol	173	4	No
	17β-Estradiol	Set at 100	Reference estrogen	Yes
	Estrone	48	4	No
	17α-Estradiol	26.5	2	No
	Coumestrol	1.9	2	Yes
	Tamoxifen citrate	1.62	1	No
≤1 to 0.1	Clomiphene citrate	0.72	1	No
	Norethynodrel	0.22	2	Yes
	Bisphenol B	0.12	2	No
<0.1 to 0.01	Bisphenol A	0.056	5	Yes
	4-Nonylphenol	0.033	10	No
	Kaempferol	0.025	1	Yes
	Daidzein	0.023	1	No
<0.01 to 0.001	4-Cumylphenol	0.005	1	No
<0.001 to 0.0001	Ethyl 4-hydroxybenzoate	0.0006	1	No
	Morin	0.0005	1	Yes
	Progesterone	0.0003	1/3*	Yes
	2-*sec*-Butylphenol	0.0003	1	No
	Phenolphthalin	0.0002	1	No
Negative	Corticosterone	Negative (100 μM)	1	No
	2,4,5-Trichloro-phenoxyacetic acid	Negative (1000 μM)	1	No

*The substance was positive in one of three tests.

about the disposal of the PCB congeners (concentrations in excess of 50 ppm require special disposal procedures), inclusion of this chemical class should be considered further.

Eight substances presently being tested by Battelle were not included in the validation list because of limited published data on their activity in the RUC assay (**Appendix D**). These are tamoxifen citrate, clomiphene citrate, 4-cumylphenol, ethyl-4-hydroxybenzoate, 2-*sec*-butylphenol, phenolphthalin, trichloroacetic acid, bisphenol B, corticosterone, and

2,4,5-trichlorophenoxyacetic acid. Rather than tamoxifen citrate, tamoxifen is recommended (the RBA values are similar) because it has been tested 21 times. 4-Nonylphenol was not selected, as much of the published reports used an undefined nonylphenol or a mixture of nonylphenol isomers. Among the substances with the highest binding affinity (RBA values ≥10), both 17β-ethinyl estradiol and *meso*-hexestrol were considered for inclusion in the proposed list of substances to be used in validation studies, but since DES and 4-hydroxytamoxifen had been tested in a wider range of assays, they were selected. Genistein was selected over daidzein in the 1 to 0.1 RBA value range because it had been tested more frequently. Although morin had only been tested once, it was included in the recommended list since it was considered desirable to have one representative flavone, where possible, in each RBA value range. Corticosterone and 2,4,5-trichlorophenoxyacetic acid, the two substances categorized as negative for ER binding in the Battelle list, were excluded from the recommended list due to limited data (i.e., the highest dose tested for corticosterone in any study was 100 μM; there was only a single study on 2,4,5-trichlorophenoxyacetic acid).

In a validation study, it is important to include substances that cover the range of possible responses without necessarily having the same numbers of substances in each of the artificially defined categories. However, for balance, it would seem that it would be desirable to have equal numbers of substances in each RBA category. When available, the results from the Battelle study might be used to modify the recommended list.

12.5 Conclusions and Recommendations

Currently, there are no published guidelines for conducting *in vitro* ER binding studies, and no formal validation studies to assess the reliability or performance of ER binding assays have been performed. To support the further development and characterization of *in vitro* ER binding assays, minimum procedural standards for such assays and a recommended list of test substances for use in validation studies are provided. The minimum procedural standards and recommended test substances are based on a comparative evaluation of the 14 *in vitro* ER binding assays summarized and evaluated in this BRD. The RUC assay, which has been the most widely used method for identifying substances with ER binding activity, is proposed as the standard against which new tests should be evaluated.

The minimum procedural standards consider methods for determining the K_d of the reference estrogen, methods for test substance preparation, the concentration range of the test substance to evaluate (including the limit dose), the use of solvent and positive controls, the number of replicates to use per test substance concentration, dose spacing, data analysis, assay acceptance criteria, evaluation and interpretation of results, minimal information to include in the test report, and the potential need for replicate studies. These minimum procedural standards are provided to ensure that *in vitro* ER binding studies will be conducted to the same minimal standards.

A suggested general protocol for measuring ER binding using the RUC assay was developed based on a submitted U.S. EPA protocol. Aspects of the RUC assay protocol presented included preparation of rat uterine cytosol, standardization of the assay, the saturation radioligand binding assay, the ER competitive binding assay, considerations for standardizing ER binding assays, ER competitive binding assay methodology, preparation of TEDG assay buffer, preparation of the radiolabeled reference estrogen, preparation of unlabeled reference estrogen, selection of ER concentration and assay volume, preparation of the reference estrogen for the standard curve and nonspecific binding measurements, preparation of test substances, preparation of ER assay tubes, preparation of the HAP slurry, separation of ER-bound radiolabeled 17β-estradiol from free labeled and unlabeled 17β-estradiol, extraction and quantification of the radiolabeled reference estrogen bound to ER, data analysis, and report specifications. It is hoped that such guidance will help investigators in their development of laboratory specific protocols for conducting validation studies on *in vitro* ER binding assays.

A number of factors were considered in developing a list of substances to be used in validation efforts, including the number of times the substance had been tested in the RUC assay, the median RBA value of the substance in the RUC assay, and the extent of concordance of the RUC median RBA value with values obtained for the same substance in other *in vitro* ER binding assays. Because the number of substances tested by multiple laboratories in the RUC assay was insufficient to generate the desired number of substances for consideration, selection of additional substances was based on the availability and concordance of multiple test data among the 13 other *in vitro* ER binding assays considered in this BRD. The selected substances were

sorted according to their median RBA values, over seven orders of magnitude, ranging from 400 to 0.0001. Weakly-binding substances (RBA values <0.001) were difficult to identify because they were not always consistently positive in tests within an assay or using different assays. Also included were substances classified as "negative" for ER binding based on the lack of a positive response in multiple assays when tested at doses of at least 1 mM. Five substances were selected for each RBA category and three for the negative category group. To ensure that each RBA category contained a representative sampling of chemical classes, selection was based on the chemical class to which the substance belongs, whether it was representative of a chemical class used in commerce or found in the environment, and whether the substance is commercially available. The latter criterion was based on whether the substance could be located in a chemical supply catalogue.

The resulting list of 33 substances was compared with the U.S. EPA list of 22 substances to be tested in an RUC assay procedure by Battelle. The U.S. EPA list lacks substances in certain chemical classes, such as PCBs and organochlorines, which have been demonstrated to bind to the ER. Since these two chemical classes are ubiquitous in the environment, representative substances were included in the proposed list of validation substances. Eight of the substances on the U.S. EPA list were not considered because of limited published data on their activity in the RUC assay.